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PATENTS



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Charlotte A. Kensil

Serial No.: To Be Assigned

Filed: Herewith

Title: COMPOSITIONS OF CPG AND SAPONIN ADJUVANTS
AND USES THEREOF

Atty Docket No.: 106941.181



Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

TRANSMITTAL LETTER

Sir:

Enclosed herewith for appropriate action by the United States Patent and Trademark Office are the following documents:

1. Utility Patent Application Transmittal (37 CFR 1.53(b)), including:

- 23 Pages of Specification
- 7 Pages of Claims
- 1 Page of Abstract
- 9 Sheets of Informal Drawings (Figs. 1-9);

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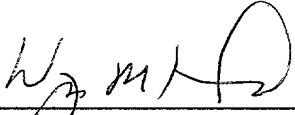
Charlotte A. Kensil
Serial No.: TBA
Page 2

2. Combined Declaration and Power of Attorney (Unsigned); and,
3. Return Postcard.

This patent application is being submitted under 37 CFR § 1.53(b) and 35 USC § 111, without the filing fee.

Respectfully submitted,

Dated: August 6, 1999



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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. § 1.53(b))

Attorney Docket No. 106941.181

First Inventor or Application Identifier Charlotte A. Kensil

Title COMPOSITIONS OF CPG AND SAPONIN ADJUVANTS
AND USES THEREOF

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APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☐ * Fee Transmittal Form (e.g., PTO/SB/17)
(Submit an original and a duplicate for fee processing)
2. ☒ Specification [Total Pages 31]
(preferred arrangement set forth below)
 - Descriptive title of the invention
 - Cross References to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to Microfiche Appendix
 - Background of the invention
 - Brief Summary of the invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
3. ☒ Drawing(s) (35 U.S.C. 113) [Total Sheets 9]
4. Oath or Declaration [Total Pages]
 - a. ☒ Newly executed (original or copy) (unsigned)
 - b. ☐ Copy from a prior application (37 C.F.R. § 1.63(d))
(for continuation/divisional with Box 16 completed)
 - i. ☐ DELETION OF INVENTOR(S)
Signed statement attached deleting
inventor(s) named in the prior application,
see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).

* NOTE FOR ITEMS 1 & 13: IN ORDER TO BE ENTITLED TO PAY SMALL ENTITY
FEES, A SMALL ENTITY STATEMENT IS REQUIRED (37 C.F.R. § 1.27), EXCEPT
IF ONE FILED IN A PRIOR APPLICATION IS RELIED UPON (37 C.F.R. § 1.28).

ADDRESS TO: Assistant Commissioner for Patents
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5. ☐ Microfiche Computer Program (Appendix)
6. Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)
 - a. ☐ Computer Readable Copy
 - b. ☐ Paper Copy (identical to computer copy)
 - c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

7. ☐ Assignment Papers (cover sheet & document(s))
8. ☐ 37 C.F.R. § 3.73(b) Statement ☐ Power of
(when there is an assignee) ☐ Attorney
9. ☐ English Translation Document (if applicable)
10. ☐ Information Disclosure ☐ Copies of IDS
Statement (IDS)/PTO-1449 ☐ Citations
11. ☐ Preliminary Amendment
12. ☒ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)
13. ☐ * Small Entity ☐ Statement filed in prior application,
Statement(s) ☐ Status still proper and desired
(PTO/SB/09-12)
14. ☐ Certified Copy of Priority Document(s)
(if foreign priority is claimed)
15. ☐ Other:

16. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No: _____ / _____

Prior application information: Examiner _____ Group / Art Unit: _____

For CONTINUATION or DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 4b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

17. CORRESPONDENCE ADDRESS

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COMPOSITIONS OF CPG AND SAPONIN ADJUVANTS AND USES THEREOF

Inventor: Charlotte R. Kensil
(Attorney Docket No. 106941.181)

RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 60/128,608, filed April 8, 1999, and of U.S. Provisional Application No. 60/095,913, filed August 10, 1998, the contents of which are both incorporated herein by reference.

FIELD OF THE INVENTION

The present invention is in the field of immune adjuvants and vaccines. The compositions of the invention stimulate immunity, enhance cell-mediated immunity, and enhance antibody production.

BACKGROUND OF THE INVENTION

Adjuvant saponins have been identified and purified from an aqueous extract of the bark of the South American tree, *Quillaja saponaria* Molina. Among the 22 saponin peaks which were separable, the more predominant purified saponins have been identified as QS-7, QS-17, QS-18, and QS-21, also known as QA-7, QA-17, QA-18, and QA-21, respectively. These saponins have been substantially purified by various methods including high pressure liquid chromatography ("HPLC"), low pressure liquid silica chromatography, and hydrophilic interactive chromatography ("HILIC"). The substantially pure saponins have been found to be useful as immune adjuvants for enhancing immune responses in individuals. (Kensil, et al., U.S. Patent

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No. 5,057,540; Kensil, et al., *J. Immunol.* 148:2357 (1991); Marciani, et al., *Vaccine* 9:89 (1991).)

Recently, oligonucleotides containing the unmethylated cytosine-guanine ("CpG") dinucleotide in a particular sequence context or motif have been shown to be potent stimulators of several types of immune cells *in vitro*. (Weiner, et al., *Proc. Natl. Acad. Sci.* 94:10833 (1997).) An immunostimulatory oligonucleotide comprising an unmethylated CpG motif is an dinucleotide within the oligonucleotide that consistently triggers an immunostimulatory response and release of cytokines. CpG motifs can stimulate monocytes, macrophages, and dendritic cells that can produce several cytokines, including the T helper 1 ("Th 1") cytokine interleukin ("IL") 12. (Carson, et al., *J. Exp. Med.* 186:1621 (1997).) This effect causes the induction of IFN- γ secretion by natural killer cells, which in turn, activates macrophages and enhances immunoglobulin isotype switching to IgG2a, a hallmark of T helper cell immunity and differentiation. (Chu, et al., *J. Exp. Med.* 186:1623 (1997).) Klinman, et al., have shown that a DNA motif consisting of an unmethylated CpG dinucleotide flanked by two 5' purines (GpA or ApA) and two 3' pyrimidines (TpC or TpT) optimally stimulated B cells to produce IL-6 and IL-12 and stimulated CD4+ T cells to produce IL-6 and IFN- γ both *in vitro* and *in vivo*. (Klinman, et al., *Proc. Natl. Acad. Sci.*, 93:2879 (1996).) Davis, et al., the contents of which are incorporated herein by reference, discovered that nucleic acids containing at least one unmethylated CpG dinucleotide may affect the immune response of a subject (Davis, et al., WO 98/40100, PCT/US98/04703).

SUMMARY OF THE INVENTION

Since immunity plays an important role in the protective response to infection with certain microbial agents, a need exists to characterize other novel adjuvants that may safely induce immunity. Such adjuvants may be potentially incorporated in future human vaccines. Surprisingly, a combination of an oligonucleotide comprising at least one unmethylated CpG dinucleotide and a saponin adjuvant was found to be a powerful stimulator of cell-mediated immunity compared to either adjuvant alone. Antibody titers (antigen-specific) in response to vaccination were significantly higher for vaccines comprising a CpG-containing oligonucleotide/saponin adjuvant combination compared to either saponin or CpG alone and represented a positive synergistic adjuvant effect. Together, these results establish that an immune adjuvant composition comprising an immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide and a saponin adjuvant is a candidate adjuvant composition for vaccines to induce immunity. Accordingly, the present invention provides novel vaccine compositions which comprise an immunostimulatory oligonucleotide, a saponin adjuvant, and an antigen. Methods for increasing the immune response to an antigen by administering the inventive vaccine compositions and/or immune adjuvant compositions are other embodiments described herein.

DESCRIPTION OF THE FIGURES

Figure 1 depicts a graph showing the enhancement of a cell-mediated immune response by QS-21 and CpG oligonucleotide/QS-21 combination, as evidenced by the CTL induction.

Figure 2 provides a graph showing the enhancement of a cell-mediated immune response by QS-21 and CpG oligonucleotide/QS-21 combination, as evidenced by the CTL induction.

Figure 3 shows a bar graph of enhanced antibody production, particularly the antibody subclasses such as IgG2a that are influenced by Th 1 cytokines.

Figure 4 shows a bar graph of IgG1 titers specific for pneumococcal Type 14 polysaccharide with the various formulations and for combinations of QS-21 and CpG oligonucleotide in mouse sera collected 21 days after a first immunization given on day 0.

Figure 5 illustrates a bar graph of IgG2a titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and CpG oligonucleotide in mouse sera collected 21 days after a first immunization given on day 0.

Figure 6 provides a bar graph of IgG3 titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and CpG oligonucleotide in mouse sera collected 21 days after a first immunization given on day 0.

Figure 7 depicts a bar graph of IgG1 titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and CpG oligonucleotide in mouse sera collected 14 days after a second immunization given 28 days after the first immunization.

Figure 8 provides a bar graph of IgG2a titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and CpG oligonucleotide in mouse sera collected 14 days after a second immunization given 28 days after the first immunization.

Figure 9 shows a bar graph of IgG3 titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and CpG oligonucleotide in mouse sera collected 14 days after a second immunization given 28 days after the first immunization.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "saponin" as used herein includes glycosidic triterpenoid compounds which produce foam in aqueous solution, have hemolytic activity in most cases, and possess immune adjuvant activity. The invention encompasses the saponin per se, as well as natural and pharmaceutically acceptable salts and pharmaceutically acceptable derivatives. The term "saponin" also encompasses biologically active fragments thereof.

The saponins of the present invention may be obtained from the tree *Quillaja saponaria* Molina. (Dalsgaard, *Acta Veterinaria Scandinavica*, 69:1 (1978).) A partially

purified saponin enriched extract, prepared as described by Dalsgaard, ("Quil-A") has adjuvant activity. Such an extract can be further separated. Among the 22 saponin peaks which were separable, the more predominant purified saponins have been identified as QS-7, QS-17, QS-18, and QS-21, also known as QA-7, QA-17, QA-18, and QA-21, respectively. (Kensil, et al., U.S. Patent No. 5,057,540.) These saponins have been substantially purified by various methods including HPLC, low pressure liquid silica chromatography, and HILIC.

As described in Kensil, et al., U.S. Patent No. 5,057,540, the contents of which are fully incorporated by reference herein, the adjuvant activity of such saponins may be determined by any of a number of methods known to those of ordinary skill in the art. The increase in antibody titer of antibody against specific antigen upon administration of an adjuvant may be used as a criteria for adjuvant activity. (Bomford, *Int. Archs. Allergy Appl. Immun.* 77:409 (1985).) Briefly, one such test involves injecting CD-1 mice intradermally with an antigen (for instance, *i.e.*, bovine serum albumin, ("BSA")) mixed with varying amounts of the potential adjuvant. Sera was harvested from the mice two weeks later and tested by ELISA for anti-BSA antibody.

Another such test involves injecting inbred mice such as C57BL/6 or Balb/c by subcutaneous route with a protein antigen such as ovalbumin ("OVA") or a polysaccharide antigen such as pneumococcal polysaccharide, mixed with the potential adjuvant. Sera harvested from the mice after one, two, or three immunizations could be harvested and tested by ELISA for antigen-specific antibody

(total immunoglobulin) or for specific mouse IgG subclasses such as IgG1 or IgG2a. Another such test involves injecting C57BL/6 mice with OVA, harvesting spleens after one, two, or three immunizations, stimulating splenocytes with antigen, and then assaying for cytolytic T lymphocyte activity ("killing") of OVA-peptide-expressing target cells. Alternative, a proliferative response could be measured in an *in vitro* assay by measuring the uptake of ³H-thymidine by antigen-stimulated splenocytes obtained from immunized animals.

"QS-21" designates the mixture of components QS-21-V1 and QS-21-V2 which appear as a single peak on reverse phase HPLC on Vydac C4 (5 µm particle size, 300Å pore, 4.6 mm ID x 25 cm length) in 40 mM acetic acid in methanol/water (58/42, v/v). The component fractions are referred to specifically as QS-21-V1 and QS-21-V2 when describing experiments performed on the further purified components.

According to Kensil, et al., U.S. Patent No. 5,583,112, the contents of which are fully incorporated by reference herein, the carboxyl group on the glucuronic acid of *Quillaja saponaria* Molina can be conjugated to a protein, a peptide, or a small molecule containing a primary amine. Thus, the present invention relates to a chemically modified saponin adjuvant or a fraction thereof obtainable from a crude *Quillaja saponaria* Molina extract, wherein the chemically modified saponin or fraction thereof comprises at least one of QS-17, QS-18, QS-21, QS-21-V1, and QS-21-V2, and wherein the modified saponin retains adjuvant activity.

The term "partially pure" means saponins partially separated from compounds normally associated with the saponin in its natural state.

The term "substantially pure" means substantially free from compounds normally associated with the saponin in its natural state and exhibiting constant and reproducible chromatographic response, elution profiles, and biologic activity. The term "substantially pure" is not meant to exclude artificial or synthetic mixtures of the saponin with other compounds.

The present invention may also employ immunostimulatory saponins isolated from other plant species. For example, a saponin from *Dolichos lablab* has been shown to be useful as an adjuvant (Katayan, et al., *Vaccine* 17:2733 (1999)).

The term "immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide" means an oligonucleotide that has been shown to activate the immune system. The immunostimulatory oligonucleotide may, preferably, comprise at least one unmethylated CpG dinucleotide. A "CpG motif" is a stretch of DNA comprising one or more CpG dinucleotides within a specified sequence. The oligonucleotide comprising the CpG motif may be as short as 5-40 base pairs in length. The immunostimulatory oligonucleotide containing the CpG motif may be a monomer or part of a multimer. Alternatively, the CpG motif may be a part of the sequence of a vector that also presents a DNA vaccine. It may be single-stranded or double-stranded. It may be prepared synthetically or produced in large scale in plasmids. One embodiment of the invention covers the immunostimulatory oligonucleotide which contains a CpG motif having the formula 5' X_1 CGX₂3', wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine. In a

preferred embodiment, the CpG motif comprises TCTCCCAGCGTGCGCCAT (also known as "1758") or TCCATGACGTTTCCTGACGTT (also known as "1826").

DNA containing unmethylated CpG dinucleotide motifs in the context of certain flanking sequences has been found to be a potent stimulator of several types of immune cells *in vitro*. (Ballas, et al., *J. Immunol.* 157:1840 (1996); Cowdrey, et al., *J. Immunol.* 156:4570 (1996); Krieg, et al., *Nature* 374:546 (1995).) Depending on the flanking sequences, certain CpG motifs may be more immunostimulatory for B cell or T cell responses, and preferentially stimulate certain species. When a humoral response is desired, preferred immunostimulatory oligonucleotides comprising an unmethylated CpG motif will be those that preferentially stimulate a B cell response. When cell-mediated immunity is desired, preferred immunostimulatory oligonucleotides comprising at least one unmethylated CpG dinucleotide will be those that stimulate secretion of cytokines known to facilitate a CD8+ T cell response.

The immunostimulatory oligonucleotides of the invention may be chemically modified in a number of ways in order to stabilize the oligonucleotide against endogenous endonucleases. For example, the oligonucleotides may contain other than phosphodiester linkages in which the nucleotides at the 5' end and/or 3' end of the oligonucleotide have been replaced with any number of non-traditional bases or chemical groups, such as phosphorothioate-modified nucleotides. The immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide may preferably be modified with at least one such phosphorothioate-modified nucleotide. Oligonucleotides with phosphorothioate-modified linkages may

be prepared using methods well known in the field such as phosphoramidite (Agrawal, et al., *Proc. Natl. Acad. Sci.* 85:7079 (1988)) or H-phosphonate (Froehler, et al., *Tetrahedron Lett.* 27:5575 (1986)). Examples of other modifying chemical groups include alkylphosphonates, phosphorodithioates, alkylphosphorothioates, phosphoramidates, 2-O-methyls, carbamates, acetamidates, carboxymethyl esters, carbonates, and phosphate triesters. Oligonucleotides with these linkages can be prepared according to known methods (Goodchild, *Chem. Rev.* 90:543 (1990); Uhlmann, et al., *Chem. Rev.* 90:534 (1990); and Agrawal, et al., *Trends Biotechnol.* 10:152 (1992)).

The term "immune adjuvant" as used herein refers to compounds which, when administered to an individual or tested *in vitro*, increase the immune response to an antigen in the individual or test system to which the antigen is administered. Preferably, such individuals are mammals, and more preferably, the mammals are humans, however, the invention is not intended to be so limiting. Any animal which may experience the beneficial effects of the vaccines of the invention are within the scope of animals which may be treated according to the claimed invention. Some antigens are weakly immunogenic when administered alone, *i.e.*, inducing no or weak antibody titers or cell-mediated immune response. An immune adjuvant may enhance the immune response of the individual by increasing antibody titers and/or cell-mediated immunity. The adjuvant effect may also lower the dose of the antigen effective to achieve an immune response in the individual.

In a first aspect of the invention, an immune adjuvant composition comprising a saponin adjuvant and an immunostimulatory oligonucleotide may be administered. More preferably, such immune adjuvant composition may increase the immune response to an antigen in an individual or a test system to which the antigen is administered. Preferably, the saponin adjuvant is a saponin from *Quillaja saponaria* Molina. More preferably, the saponin adjuvant is a partially pure or substantially pure saponin from *Quillaja saponaria* Molina. Preferably, the partially pure saponin may comprise QS-7, QS-17, QS-18, and/or QS-21 and may comprise other saponins. Preferably, the substantially pure saponin adjuvant is QS-7, QS-17, QS-18, or QS-21. Most preferably, the substantially pure saponin adjuvant is QS-21. Alternatively, the immune adjuvant composition may comprise more than one substantially pure saponin adjuvant with the immunostimulatory oligonucleotide. In a further preferred embodiment, the saponin adjuvant may cover a chemically modified saponin adjuvant or a fraction thereof obtainable from a crude *Quillaja saponaria* Molina extract, wherein the chemically modified saponin or fraction thereof comprises at least one of QS-17, QS-18, QS-21, QS-21-V1, and QS-21-V2, and wherein the chemically modified saponin retains adjuvant activity. The immunostimulatory oligonucleotide, preferably, comprises at least one unmethylated CpG dinucleotide. The CpG dinucleotide is preferably a monomer or multimer. Another preferred embodiment of the CpG motif is as a part of the sequence of a vector that also presents a DNA vaccine. Yet another embodiment of the immune adjuvant composition is directed to the immunostimulatory oligonucleotide, wherein the

immunostimulatory oligonucleotide is modified. The particular modification may comprise at least one phosphorothioate-modified nucleotide. Further, the immunostimulatory oligonucleotide having at least one unmethylated CpG dinucleotide may comprise a CpG motif having the formula 5' X_1 CG X_2 3', wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine. The CpG motif may preferentially be TCTCCCAGCGTGCGCCAT or TCCATGACGTTCTGACGTT.

In a second aspect, the invention is directed to a method for increasing the immune response to an antigen in an individual or a test system to which the antigen is administered comprising administering an effective amount of an immune adjuvant composition comprising a saponin adjuvant and an immunostimulatory oligonucleotide further. Preferably, the saponin adjuvant is a saponin from *Quillaja saponaria* Molina. More preferably, the saponin adjuvant is a partially pure or a substantially pure saponin from *Quillaja saponaria* Molina. The method may also embody an immune adjuvant composition comprising more than one substantially pure saponin adjuvant and immunostimulatory oligonucleotide. The substantially pure saponin adjuvant is preferably QS-7, QS-17, QS-18, or QS-21. Most preferably, the substantially pure saponin adjuvant is QS-21. In a further preferred embodiment, the saponin adjuvant may cover a chemically modified saponin adjuvant or a fraction thereof obtainable from a crude *Quillaja saponaria* Molina extract, wherein the chemically modified saponin or fraction thereof comprises at least one of QS-17, QS-18, QS-21, QS-21-V1, and QS-21-V2, and wherein the chemically modified saponin

retains adjuvant activity. In a preferred embodiment of the method, the immunostimulatory oligonucleotide comprises at least one unmethylated CpG dinucleotide. The CpG motif is preferably a monomer or a multimer. Another preferred embodiment of the method includes the CpG motif as a part of the sequence of a vector that presents a DNA vaccine. Yet another embodiment is directed to the method wherein the immunostimulatory oligonucleotide comprises at least one unmethylated CpG dinucleotide, and wherein furthermore, the immunostimulatory oligonucleotide may be chemically modified to stabilize the oligonucleotide against endogenous endonucleases. The modification may comprise at least one phosphorothioate-modified nucleotide. Further, the method may be directed, in part, to the immunostimulatory oligonucleotide having at least one unmethylated CpG dinucleotide comprising a CpG motif having the formula 5'X₁CGX₂3', wherein at least one nucleotide separates consecutive CpGs, and wherein X₁ is adenine, guanine, or thymine, and X₂ is cytosine, thymine, or adenine. In another preferred method, the unmethylated CpG motif is TCTCCCAGCGTGCGCCAT or TCCATGACGTTCTGACGTT.

The term "vaccine composition" herein refers to a composition capable of producing an immune response. A vaccine composition, according to the invention, would produce immunity against disease in individuals. The combination of saponin and immunostimulatory oligonucleotide of the present invention may be administered to an individual to enhance the immune response to any antigen. Preferably, the vaccine composition stimulates immunity. More preferably, the

vaccine composition enhances antibody production to an antigen and enhances a cell-mediated immune response to an antigen.

The vaccine composition of the invention may enhance antibody production to an antigen in a positive synergistic manner. The synergistic adjuvant effect of the immunostimulatory oligonucleotide and the saponin adjuvant described herein may be shown in a number of ways. For example, a synergistic adjuvant effect may be demonstrated as an increase in the maximum expected immune response. One may expect an additive effect of combining two adjuvants. Specifically, if one adjuvant, used at optimum doses, produces "X" and the other adjuvant, also used at optimum doses, produces "Y" antibody, then the combination may be expected to produce "X+Y" if the result is additive and not synergistic. A maximum level of response that is considerably higher than "X+Y" would be considered a synergistic effect and would be unexpected. A second indication of synergism would be the appearance of a substantial adjuvant effect at doses that are normally not expected to produce an adjuvant effect. A third indication of synergism would be the appearance of an immune response with earlier kinetics than expected for either adjuvant alone.

Further, typical antigens suitable for the enhanced immune response include antigens derived from any of the following: viruses, such as influenza, feline leukemia virus, feline immunodeficiency virus, HIV-1, HIV-2, rabies, measles, hepatitis B, or hoof and mouth disease; bacteria, such as anthrax, diphtheria, Lyme disease, pneumococcus, or tuberculosis; or protozoans, such as *Babesiosis bovis* or Plasmodium. The antigen may preferably be a protein, a peptide, a polysaccharide, a

lipid, a glycolipid, a phospholipid, or a nucleic acid encoding the antigenic protein or peptide of interest. The antigens may be purified from a natural source, synthesized by means of solid phase synthesis, or may be obtained by means of genetic engineering.

Accordingly, in a third aspect, the invention also encompasses a vaccine composition comprising a saponin adjuvant, an immunostimulatory oligonucleotide, and an antigen. The saponin adjuvant may be partially pure or substantially pure saponin from *Quillaja saponaria* Molina. The vaccine compositions may also comprise more than one partially pure or substantially pure saponin adjuvant, an immunostimulatory oligonucleotide further comprising at least one unmethylated CpG motif, and an antigen. Preferably, the partially pure saponin adjuvant comprises QS-7, QS-17, QS-18, and/or QS-21 and may comprise other saponins. Preferably, the substantially pure saponin adjuvant is QS-7, QS-17, QS-18, or QS-21. A further preferred embodiment encompasses saponin adjuvants wherein a chemically modified saponin adjuvant or a fraction thereof obtainable from a crude *Quillaja saponaria* Molina extract, wherein the chemically modified saponin or fraction thereof comprises at least one of QS-17, QS-18, QS-21, QS-21-V1, and QS-21-V2, and wherein the chemically modified saponin retains adjuvant activity. Most preferably, the partially pure or substantially pure saponin adjuvant in the vaccine composition is QS-21. The immunostimulatory oligonucleotide may preferably comprise at least one unmethylated CpG dinucleotide. The CpG motif may preferably be a monomer or a multimer. Another preferred embodiment of the CpG motif is as a part of the

sequence of a vector that also presents a DNA vaccine. Yet another embodiment of the vaccine composition described herein is directed to the immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide comprises a chemical modification. More particularly, the immunostimulatory oligonucleotide may be modified with at least one phosphorothioate-modified nucleotide. Further, the immunostimulatory oligonucleotide having at least one unmethylated CpG dinucleotide of the vaccine composition comprises a CpG motif having the formula 5'X₁CGX₂3', wherein at least one nucleotide separates consecutive CpGs, and wherein X₁ is adenine, guanine, or thymine, and X₂ is cytosine, thymine, or adenine. The unmethylated CpG motif according to this aspect of the invention may preferentially comprise TCTCCAGCGTGCGCCAT or TCCATGACGTTCTGACGTT.

A fourth aspect of the invention encompasses a method of stimulating immunity to an antigen in an individual comprising administering an effective amount of a vaccine composition comprising an antigen, a partially pure or substantially pure saponin adjuvant, and an immunostimulatory oligonucleotide. The method also embodies a vaccine composition comprising more than one partially pure or substantially pure saponin adjuvant, an immunostimulatory oligonucleotide, and an antigen. Preferably, the partially pure saponin adjuvant comprises QS-7, QS-17, QS-18, and/or QS-21 and may comprise other saponins. Preferably, the substantially pure saponin adjuvant comprises QS-7, QS-17, QS-18, or QS-21. Most preferably, according to this method, the partially pure or substantially pure saponin adjuvant is QS-21. The saponin adjuvant may preferably be a chemically modified

saponin adjuvant or a fraction thereof obtainable from a crude *Quillaja saponaria* Molina extract, wherein the chemically modified saponin or fraction thereof comprises at least one of QS-17, QS-18, QS-21, QS-21-V1, and QS-21-V2, and wherein the chemically modified saponin retains adjuvant activity. Preferably, the method comprises administering an immunostimulatory oligonucleotide which further comprises at least one unmethylated CpG dinucleotide. The CpG dinucleotide therein is a monomer or a multimer. Another preferred embodiment of the method includes the CpG motif as a part of the sequence of a vector that also presents a DNA vaccine. Yet another embodiment of the method disclosed herein is directed to the immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide, wherein the immunostimulatory oligonucleotide may be chemically modified to increase its stability to endogenous endonucleases. Such a modification may comprise at least one phosphorothioate-modified nucleotide. Further, the immunostimulatory oligonucleotide having at least one unmethylated CpG dinucleotide may comprise a CpG motif having the formula 5'X₁CGX₂3', wherein at least one nucleotide separates consecutive CpGs, and wherein X₁ is adenine, guanine, or thymine, and X₂ is cytosine, thymine, or adenine. In another preferred embodiment, the unmethylated CpG motif is TCTCCCAGCGTGCGCCAT or TCCATGACGTTCTGACGTT.

Other useful methods for the vaccine composition include enhancing antibody production to an antigen and enhancing cell-mediated immunity. More preferably, the vaccine composition enhances antibody production to an antigen and enhances a

cell-mediated immunity. Most preferably, the vaccine composition enhances antibody production to an antigen in a positive synergistic manner.

Administration of the compositions of the present invention may be by parenteral, intravenous, intramuscular, subcutaneous, intranasal, oral, mucosal, or any other suitable means. The dosage administered may be dependent upon the age, weight, kind of concurrent treatment, if any, and nature of the antigen administered. The initial dose may be followed up with a booster dosage after a period of about four weeks to enhance the immunogenic response. Further booster dosages may also be administered. The composition may be given as a single injection of a mixed formulation of saponin, oligonucleotide, and antigen or as separate injections given at the same site within a short period of time (*i.e.*, 0-2 days).

The effective compositions of the present invention may be employed in such forms as capsules, liquid solutions, suspensions or elixirs for oral administration, or sterile liquid forms such as solutions or suspensions. Any inert acceptable carrier may preferably be used, such as saline, or PBS, or any such acceptable carrier in which the compositions of the present invention have suitable solubility properties for use of the present invention.

EXAMPLES

A well-established animal model was used to assess whether formulations of CpG oligonucleotide and QS-21 together could function as an immune adjuvant. In brief, experiments were set up to compare QS-21 to the recently reported adjuvant

CpG motif. A CpG sequence (*e.g.*, 1758), reported to serve as an adjuvant for a B-cell lymphoma idiotype-KLH vaccine in mice, was selected. One experiment evaluated whether the CpG motif, alone or in combination with QS-21, can serve as an adjuvant for a subunit vaccine, *e.g.*, OVA, in mice in inducing CTL responses. This work included a dose range experiment with CpG to determine the optimum dose.

In addition to comparing CpG and QS-21 as adjuvants, a second experiment combining CpG oligonucleotide with suboptimal doses of QS-21 (*e.g.*, 1.25 µg) was conducted to assess whether CpG oligonucleotide can affect the adjuvant effect of QS-21.

Also, an experiment was performed to determine whether the CpG and QS-21 combination could enhance antibody production, specifically the isotype profile of a antigen-specific antibody response.

Finally, a series of experiments were performed to determine whether a combination of CpG oligonucleotide and saponin would enhance antibody production in a positive synergistic manner. This work used vaccine formulations of pneumococcal Type 14 polysaccharide and QS-21 and CpG oligonucleotide and evaluated specific antibody titers harvested from mice on days 21 and 42 after immunization on days 0 and 28. Another CPG sequence (*e.g.*, 1826), reported to serve as an adjuvant for hen egg lysozyme in mice, was selected.

The experiments were done using materials from the following suppliers: OVA, Grade VI (Sigma); pneumococcal Type 14 polysaccharide (ATCC); QS-21 (Aquila); CpG oligonucleotides included the phosphorothiate-modified sequence 1758

TCTCCCAGCGTGCGCCA and phosphorothiate-modified sequence 1826
TCCATGACGTTCTGACGTT (Life Technologies (Gibco)).

Example 1
CTL Induced by QS-21 and CpG/QS-21

C57BL/6 mice (5 per group, female, 8-10 weeks of age) were immunized by subcutaneous route at days 1, 15, and 29. The vaccines were 25 µg OVA antigen plus the indicated doses of adjuvant in a total volume of 0.2 ml phosphate-buffered saline. The CpG motif used in this experiment was a phosphorothioate-modified oligonucleotide 1758 with a sequence of TCTCCCAGCGTGCGCCA (Weiner, et al., *Proc. Natl. Acad. Sci.* 94:10833 (1997).) Splenocytes were removed at day 42 for use as effector cells in the CTL assay. They were stimulated *in vitro* for 6 days with mitomycin C-treated E.G7-OVA cells and then used in a standard ⁵¹Cr release CTL assay. E.G7-OVA cells (loaded with ⁵¹Cr) were used as target cells. The background lysis of EL4 cells (not transfected by OVA) was subtracted from the lysis of E.G7-OVA cells to obtain a percent (%) antigen-specific lysis.

The results, as shown in Figure 1, indicate that no lysis was observed in the absence of adjuvant, with any CpG dose, or with 1.25 µg of QS-21 (suboptimal dose). However, the suboptimal dose of QS-21, in combination with CpG, induced significant CTL. The results show a substantial adjuvant effect at doses that are normally not expected to produce such an adjuvant effect. This positive synergistic effect was most notable at the higher dose of CpG (50 µg). The adjuvant effect was comparable to that achieved with the optimal 10 µg QS-21 control.

Example 2
CTL Induced by QS-21 and CpG/QS-21

Splenocytes from mice immunized as described in Figure 1 were used in a CTL assay. Splenocytes were stimulated *in vitro* with denatured OVA for six days prior to use in the CTL assay. The assay was carried out against E.G7-OVA cells as described in Example 1.

As evident from the results in Figure 2, no lysis was observed in the absence of adjuvant, with any CpG dose, or with 1.25 µg of QS-21 (suboptimal dose). However, the suboptimal dose of QS-21, in combination with CpG, induced significant CTL (comparable to the optimal 10 µg QS-21 control). The results illustrate the positive synergism between the CpG and the QS-21 that was unexpected at a suboptimal dose.

Example 3
Antigen-specific Serum IgG1 and IgG2a

Serum titers to OVA were determined by EIA on sera collected on day 42 from the mice immunized as described in Example 1. IgG subclass IgG1 and IgG2a titers were determined for individual mice (5 mice per group) and are plotted as a geometric mean titer. The IgG1 titers were highest in groups receiving QS-21 alone (at the 10 µg dose) or 10 µg QS-21 in combination with either 10 or 50 µg (approximate 10 fold enhancement over the unadjuvanted group) as seen in Figure 3. The IgG2a response was not detectable in any groups except for the combination of 10 µg QS-21 (optimal dose) with 10 or 50 µg CpG and the combination of 1.25 µg QS-

21 (suboptimal dose) with 50 µg CpG. IgG2a was not detected with any CpG dose used alone, with any QS-21 dose used alone, or in the unadjuvanted group.

Example 4
Antibody Induced by QS-21 and QS-21/CpG
to Pneumococcal Polysaccharide Antigen

BALB/c mice (5 mice per group, female, 8-10 weeks of age) were immunized by subcutaneous route at day 0 only or at days 0 and 28. The vaccines were 0.5 µg pneumococcal Type 14 polysaccharide plus the indicated doses of adjuvant in a total volume of 0.2 ml phosphate-buffered saline. The immunostimulatory motif CpG used in this experiment was a phosphorothioate-modified oligonucleotide 1826 with a sequence of TCCATGACGTTCTGACGTT (Chu, et al., *J. Exp. Med.* 186:1623-1631 (1997)). QS-21 was used at a dose of 1.25 µg or 10 µg. CpG ODN 1826 was used at a dose of only 10 µg.

Sera from mice receiving a single immunization was collected at day 21. Sera from mice receiving 2 immunizations was collected at day 42. Antibody titers specific for Type 14 polysaccharide was determined on the sera. IgG subclasses IgG1, IgG2a, and IgG3 were determined for an equivolume sera pool from the mice in each group. After a single immunization, IgG1 titers were 66 fold higher for the 10 µg QS-21/10 µg CpG combination than for QS-21 alone and were 43 fold higher than for CpG alone (Figure 4). IgG2a titers were 11 fold higher for the 10 µg QS-21/CpG combination than for either QS-21 alone or CpG alone (Figure 5). IgG3 titers were 85 fold higher for the 10 µg QS-21/CpG combination than for QS-21 alone and were 95 fold higher than for CpG alone (Figure 6).

After two immunizations, IgG1 titers were 46 fold higher for the 10 μ g QS-21/CpG combination than for QS-21 alone and were 444 fold higher than for CpG alone (Figure 7). IgG2a titers were 476 fold higher for the 10 μ g QS-21/CpG combination than for QS-21 alone and were 127 fold higher than for CpG alone (Figure 5). IgG3 titers were 67 fold higher for the 10 μ g QS-21/CpG combination than for QS-21 alone and were 243 fold higher than for CpG alone (Figure 9). The enhancement of these titers shows that this is a positive synergistic effect and is not simply an additive adjuvant effect of combining these two adjuvants. In addition, the combination of low doses of QS-21 (1.25 μ g) with 10 μ g CpG also produced IgG1 and IgG3 titers after two immunizations that were higher than those produced by either 1.25 μ g QS-21 alone, 10 μ g QS-21 alone, or 10 μ g CpG alone.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth below.

We claim:

1. A vaccine composition comprising:
 - (a) an antigen;
 - (b) a saponin adjuvant; and
 - (c) an immunostimulatory oligonucleotide.
2. The vaccine composition as claimed in claim 1, wherein the saponin adjuvant is derived from *Quillaja saponaria*.
3. The vaccine composition as claimed in claim 2, wherein the saponin adjuvant comprises a substantially pure saponin adjuvant.
4. The vaccine composition as claimed in claim 3, wherein the substantially pure saponin adjuvant comprises QS-7, QS-17, QS-18, or QS-21.
5. The vaccine composition as claimed in claim 4, wherein the substantially pure saponin adjuvant comprises QS-21.
6. The vaccine composition as claimed in claim 1, wherein the immunostimulatory oligonucleotide comprises at least one unmethylated CpG dinucleotide.
7. The vaccine composition as claimed in claim 1, wherein the immunostimulatory oligonucleotide is modified.
8. The vaccine composition as claimed in claim 1, wherein the immunostimulatory oligonucleotide is modified with at least one phosphorothioate-modified nucleotide.

9. The vaccine composition as claimed in claim 6, wherein the immunostimulatory oligonucleotide comprises a CpG motif having the formula 5'X₁CGX₂3', wherein at least one nucleotide separates consecutive CpGs, and wherein X₁ is adenine, guanine, or thymine, and X₂ is cytosine, thymine, or adenine.

10. The vaccine composition as claimed in claim 9, wherein the CpG motif comprises TCTCCCAGCGTGCGCCAT or TCCATGACG TTCCTGACGTT.

11. The vaccine composition as claimed in claim 1, wherein the composition increases the immune response to the antigen when administered to a mammal.

12. The vaccine composition as claimed in claim 1, wherein the composition increases the immune response to the antigen when administered to a human.

13. The vaccine composition as claimed in claim 1, wherein the composition increases the immune response to the antigen when administered to an animal.

14. The vaccine composition as claimed in claim 1, wherein the composition further stimulates immunity.

15. The vaccine composition as claimed in claim 1, wherein the composition further enhances antibody production to the antigen.

16. The vaccine composition as claimed in claim 1, wherein the composition further enhances antibody production to the antigen in a positive synergistic manner.

17. The vaccine composition as claimed in claim 1, wherein the composition further enhances cell-mediated immunity.

18. The vaccine composition as claimed in claim 1, wherein the antigen comprises a protein, a peptide, a polysaccharide, a lipid, a glycolipid, a phospholipid, or a nucleic acid encoding the protein or peptide.

19. An immune adjuvant composition comprising

- (a) a saponin adjuvant; and
- (b) an immunostimulatory oligonucleotide.

20. The immune adjuvant composition as claimed in claim 19, wherein the saponin adjuvant is derived from *Quillaja saponaria*.

21. The immune adjuvant composition as claimed in claim 20, wherein the saponin adjuvant comprises a substantially pure saponin adjuvant.

22. The immune adjuvant composition as claimed in claim 21, wherein the substantially pure saponin adjuvant comprises QS-7, QS-17, QS-18, or QS-21.

23. The immune adjuvant composition as claimed in claim 22, wherein the substantially pure saponin adjuvant comprises QS-21.

24. The immune adjuvant composition as claimed in claim 19, wherein the immunostimulatory oligonucleotide comprises at least one unmethylated CpG dinucleotide.

25. The immune adjuvant composition as claimed in claim 19, wherein the immunostimulatory oligonucleotide is modified.

26. The immune adjuvant composition as claimed in claim 25, wherein the immunostimulatory oligonucleotide is modified with at least one phosphorothioate-modified nucleotide.

27. The immune adjuvant composition as claimed in claim 24, wherein the immunostimulatory oligonucleotide comprises a CpG motif having the formula 5'X₁CGX₂3', wherein at least one nucleotide separates consecutive CpGs, and wherein X₁ is adenine, guanine, or thymine, and X₂ is cytosine, thymine, or adenine.

28. The immune adjuvant composition as claimed in claim 27, wherein the CpG motif comprises TCTCCCAGCGTGCGCCAT or TCCATGACGTTCTGACGTT.

29. The immune adjuvant composition as claimed in claim 19, wherein the composition increases the immune response to an antigen when administered to a mammal.

30. The immune adjuvant composition as claimed in claim 19, wherein the composition increases the immune response to an antigen when administered to a human.

31. The immune adjuvant composition as claimed in claim 19, wherein the composition increases the immune response to an antigen when administered to a animal.

32. The immune adjuvant composition as claimed in claim 27, wherein the antigen comprises a protein, a peptide, a polysaccharide, a lipid, a glycolipid, a phospholipid, or a nucleic acid encoding the protein or peptide.

33. A method for stimulating immunity to an antigen in an individual comprising administering an effective amount of a vaccine composition as claimed in claim 1.

34. The method as claimed in claim 33, wherein the saponin adjuvant is derived from *Quillaja saponaria*.

35. The method as claimed in claim 34, wherein the saponin adjuvant comprises a substantially pure saponin adjuvant.

36. The method as claimed in claim 35, wherein the substantially pure saponin adjuvant comprises QS-7, QS-17, QS-18, or QS-21.

37. The method as claimed in claim 36, wherein the substantially pure saponin adjuvant comprises QS-21.

38. The method as claimed in claim 33, wherein the immunostimulatory oligonucleotide comprises at least one unmethylated CpG dinucleotide.

39. The method as claimed in claim 33, wherein the immunostimulatory oligonucleotide is modified.

40. The method as claimed in claim 39, wherein the immunostimulatory oligonucleotide is modified with at least one phosphorothioate-modified nucleotide.

41. The method as claimed in claim 38, wherein the immunostimulatory oligonucleotide comprises a CpG motif having the formula 5' X_1 CG X_2 3', wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

42. The method as claimed in claim 41, wherein the CpG motif comprises TCTCCCAGCGTGCGCCAT or TCCATGACGTTCTGACGTT.

43. The method as claimed in claim 33, wherein the composition increases the immune response to an antigen when administered to a mammal.

44. The method as claimed in claim 33, wherein the composition increases the immune response to an antigen when administered to a human.

45. The method as claimed in claim 33, wherein the composition increases the immune response to an antigen when administered to an animal.

46. The method as claimed in claim 33, wherein the method further enhances antibody production to the antigen.

47. The method as claimed in claim 46, wherein the method further enhances antibody production in a positive synergistic manner.

48. The method as claimed in claim 33, wherein the method further enhances cell-mediated immunity.

49. A method for increasing the immune response to an antigen in an individual or a test system to which the antigen is administered comprising administering an effective amount of an immune adjuvant composition as claimed in claim 19.

50. The method as claimed in claim 49, wherein the saponin adjuvant is derived from *Quillaja saponaria*.

51. The method as claimed in claim 50, wherein the saponin adjuvant comprises a substantially pure saponin adjuvant.

52. The method as claimed in claim 51, wherein the substantially pure saponin adjuvant comprises QS-7, OS-17, QS-18, or QS-21.

53. The method as claimed in claim 52, wherein the substantially pure saponin adjuvant comprises QS-21.

54. The method as claimed in claim 49, wherein the immunostimulatory oligonucleotide comprises at least one unmethylated CpG dinucleotide.

55. The method as claimed in claim 49, wherein the immunostimulatory oligonucleotide is modified.

56. The method as claimed in claim 55, wherein the immunostimulatory oligonucleotide is modified with at least one phosphorothioate-modified nucleotide.

57. The method as claimed in claim 54, wherein the immunostimulatory oligonucleotide comprises a CpG motif having the formula 5' X_1 CG X_2 3', wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

58. The method as claimed in claim 57, wherein the CpG motif comprises TCTCCCAGCGTGCGCCAT or TCCATGACGTTCTGACGTT.

59. The method as claimed in claim 49, wherein the composition increases the immune response to an antigen when administered to a mammal.

60. The method as claimed in claim 49, wherein the composition increases the immune response to an antigen when administered to a human.

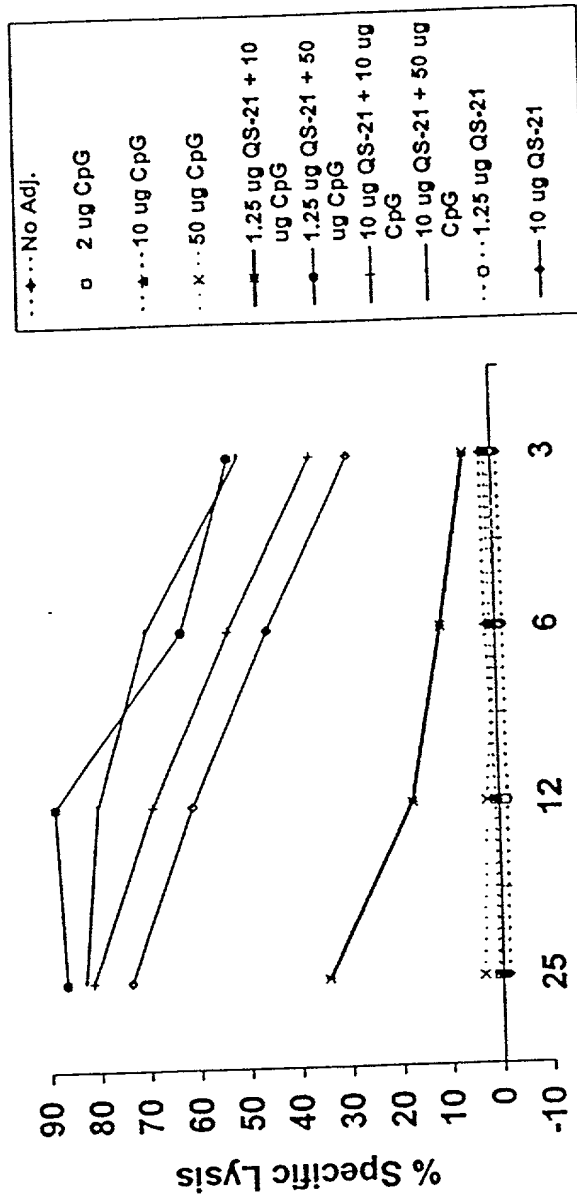
61. The method as claimed in claim 49, wherein the composition increases the immune response to an antigen when administered to an animal.

62. The method as claimed in claim 59, wherein the antigen comprises a protein, a peptide, a polysaccharide, a lipid, a glycolipid, a phospholipid, or a nucleic acid encoding the protein or peptide.

ABSTRACT

Vaccine compositions of immunostimulatory oligonucleotides and saponin adjuvants and antigens and the use thereof for stimulating immunity, enhancing cell-mediated immunity, and enhancing antibody production are disclosed. Also described are immune adjuvant compositions comprising immunostimulatory oligonucleotides and saponin adjuvants, as well as methods for increasing an immune response using the same.

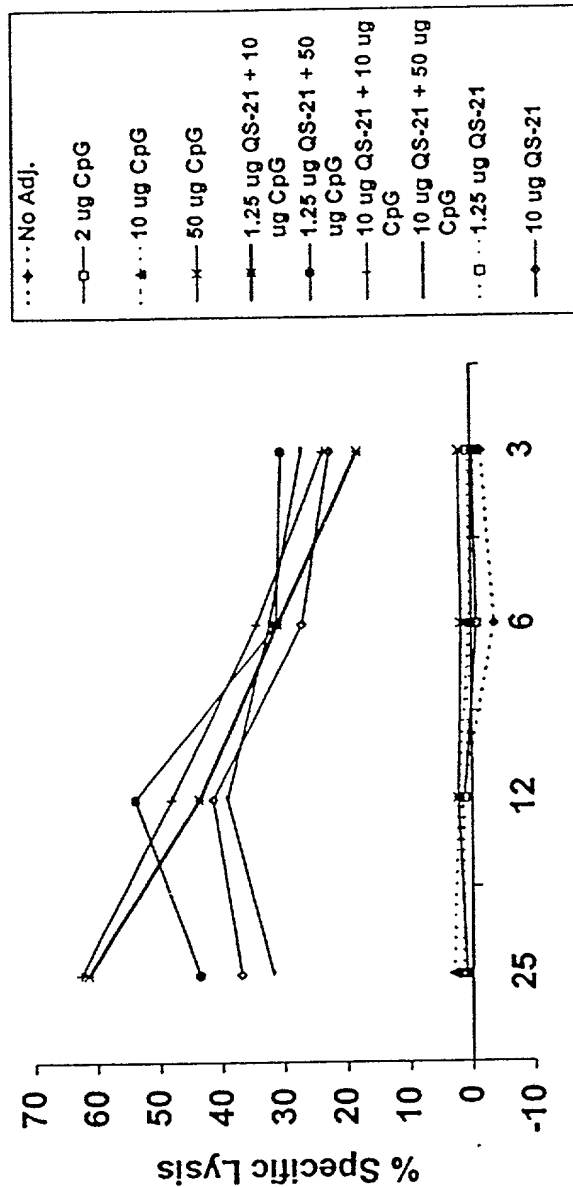
CpG vs QS-21 vs Mixtures (E.G7 stim.)



C57BL/6 mice
 3 s.c. immunizations with OVA + adj. at days 0, 14, 28
 CTL carried out with E.G7-OVA stim. splenocytes at day 42
 Targets = E.G7-OVA

Figure 1: CTL Induced by QS-21 and CpG/QS-21

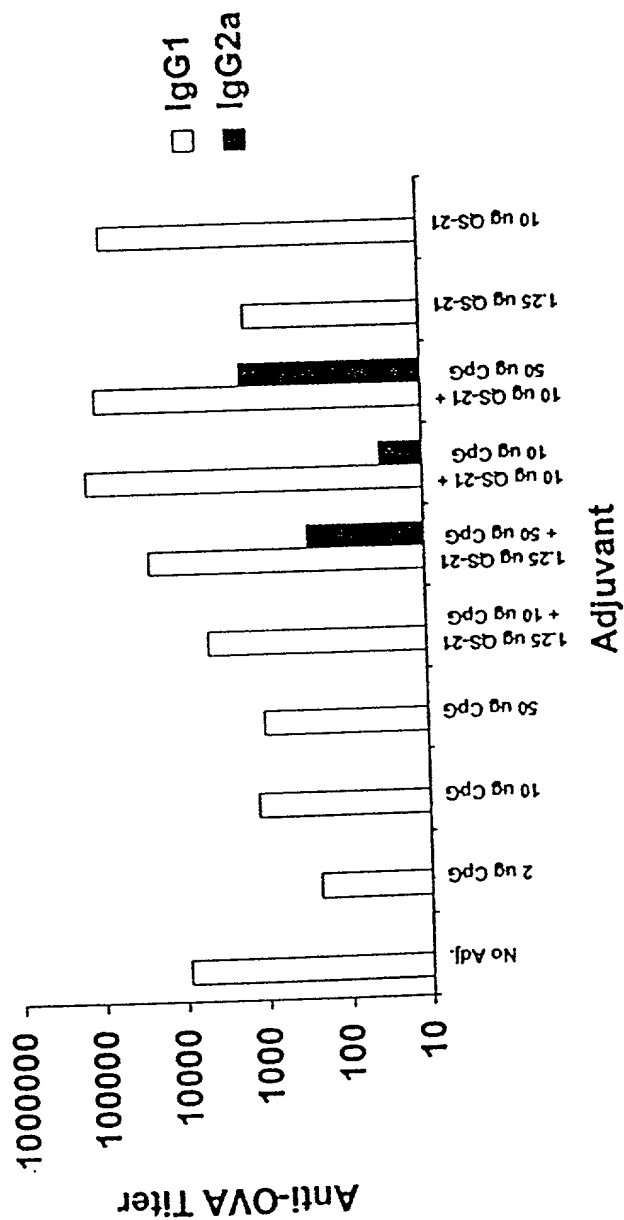
CpG Vs QS-21 Vs Mixture (Den.OVA stim)



C57BL/6 mice
3 s.c. immunizations with OVA + adj. at days 0, 14, 28
CTL carried out with E.G7-OVA stim. splenocytes at day 42
Targets = E.G7-OVA

Figure 2: CTL Induced by QS-21 and CpG/QS-21

CpG Vs QS-21 Vs Mixture (Serum IgG1, IgG2a)



C57BL/6 mice
3 s.c. immunizations with OVA + adj. at days 0, 14, 28
Sera collected at day 42 and assayed for anti-OVA IgG1 and IgG2a.

Figure 3: Antigen-specific Serum IgG1 and IgG2a

Figure 4

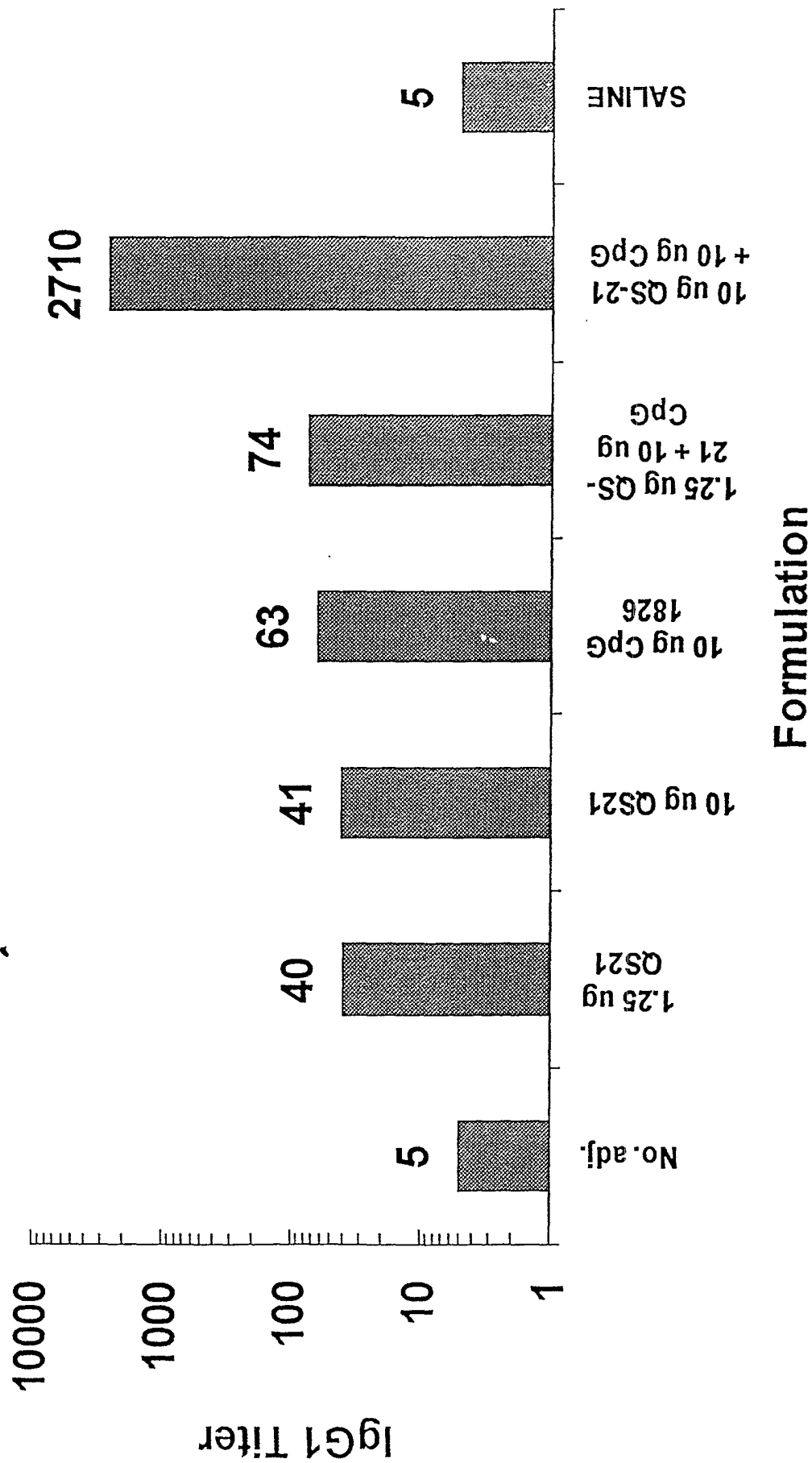


Figure 5

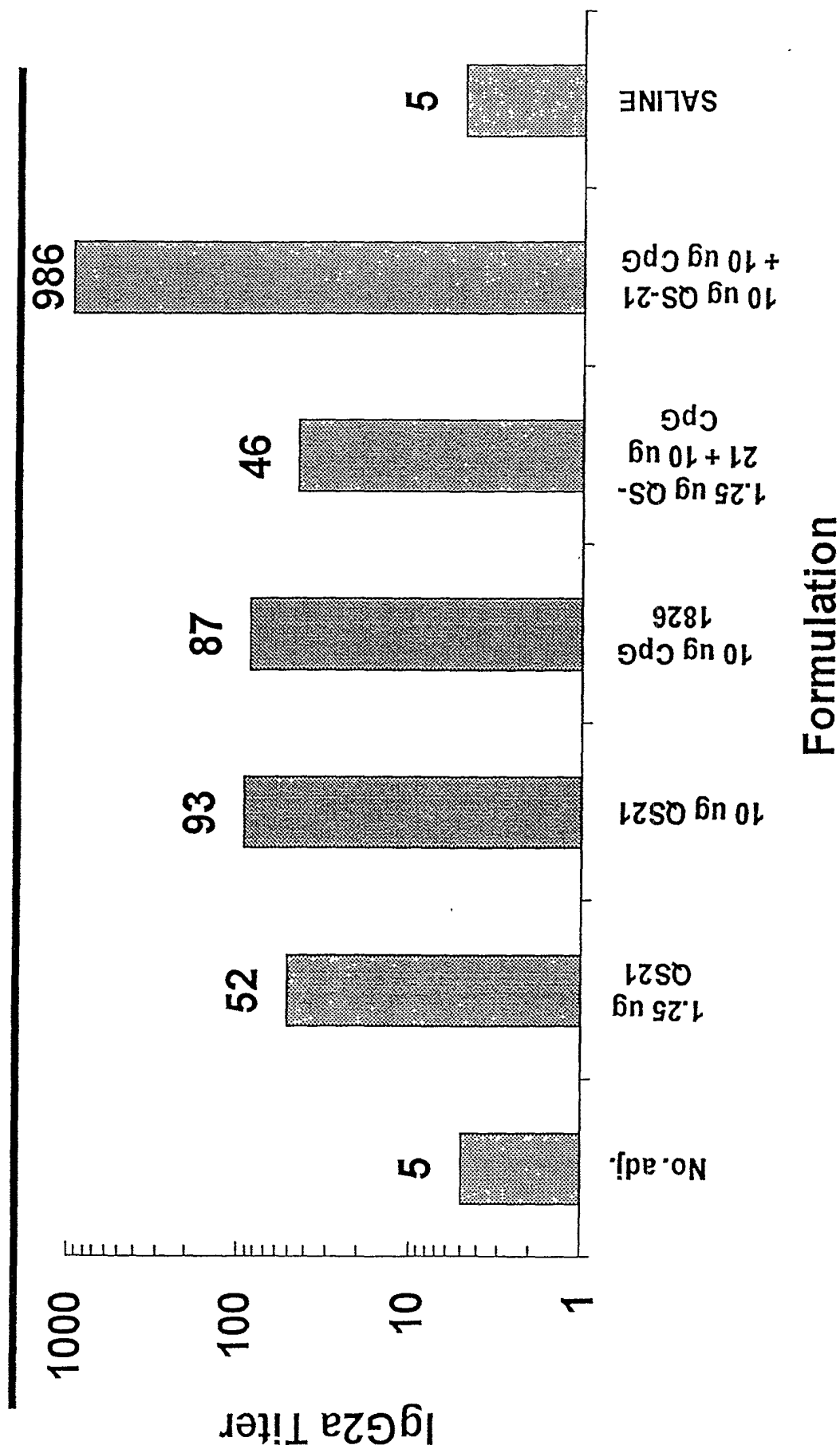


Figure 6

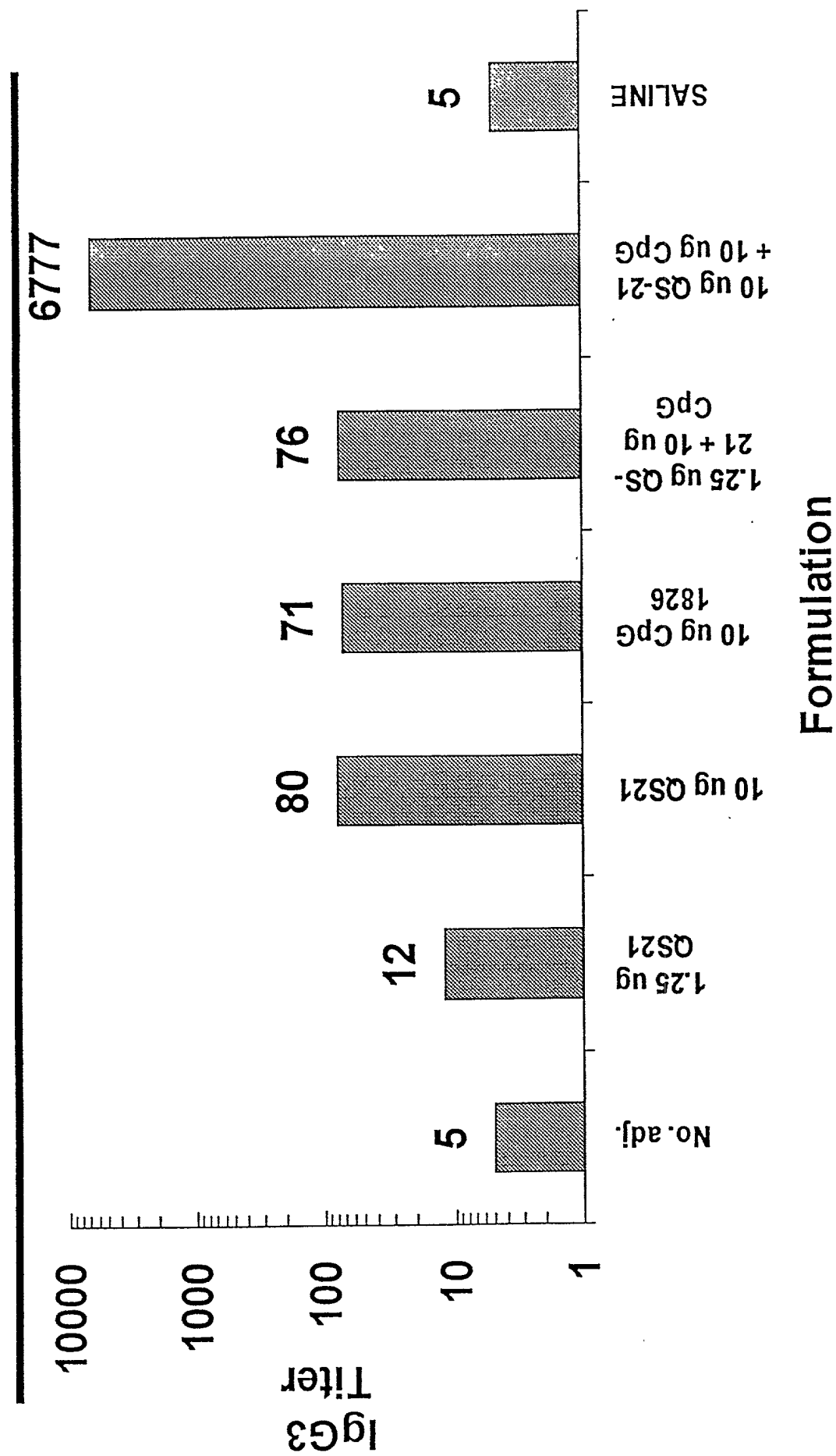


Figure 7

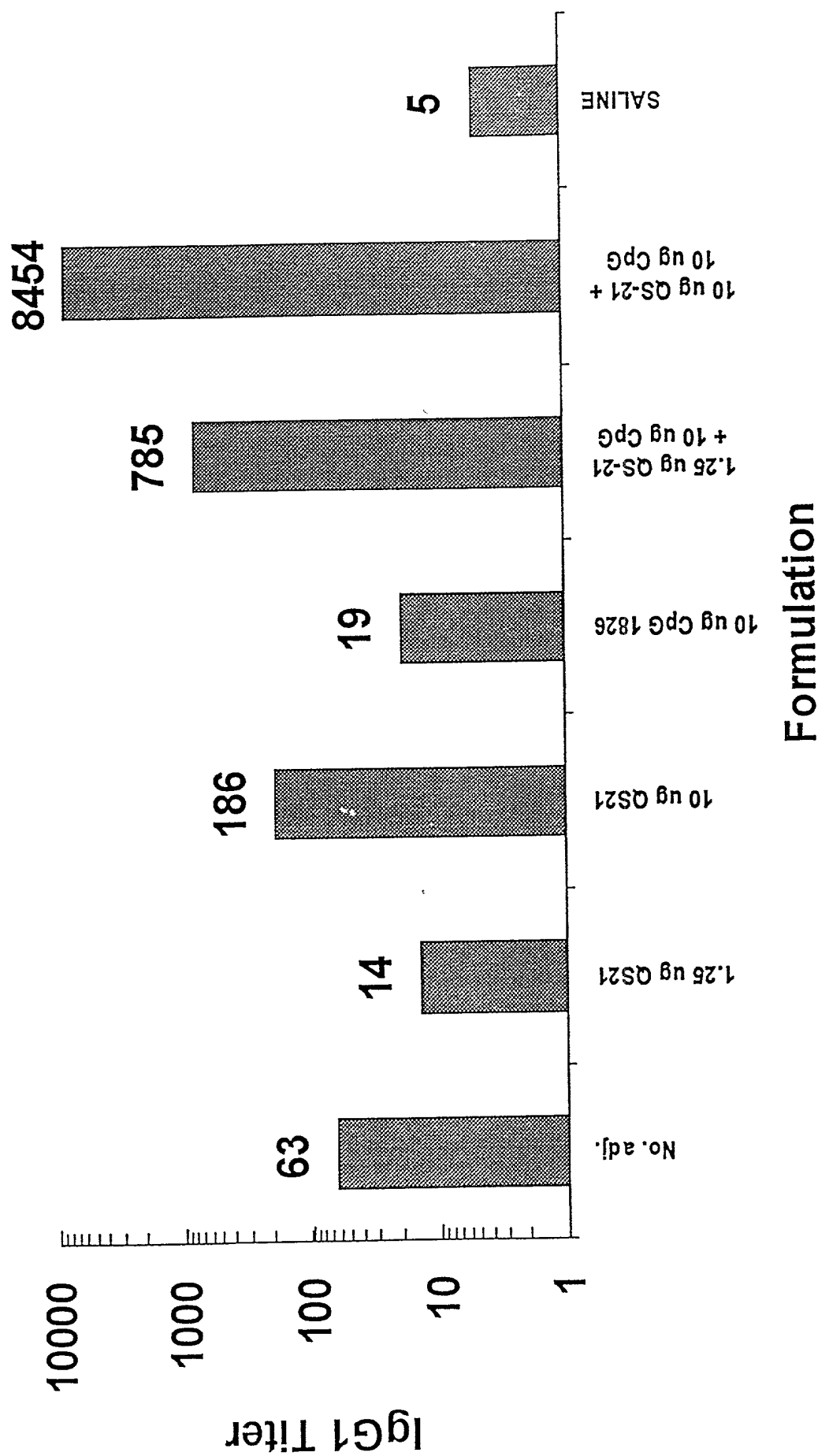


Figure 8

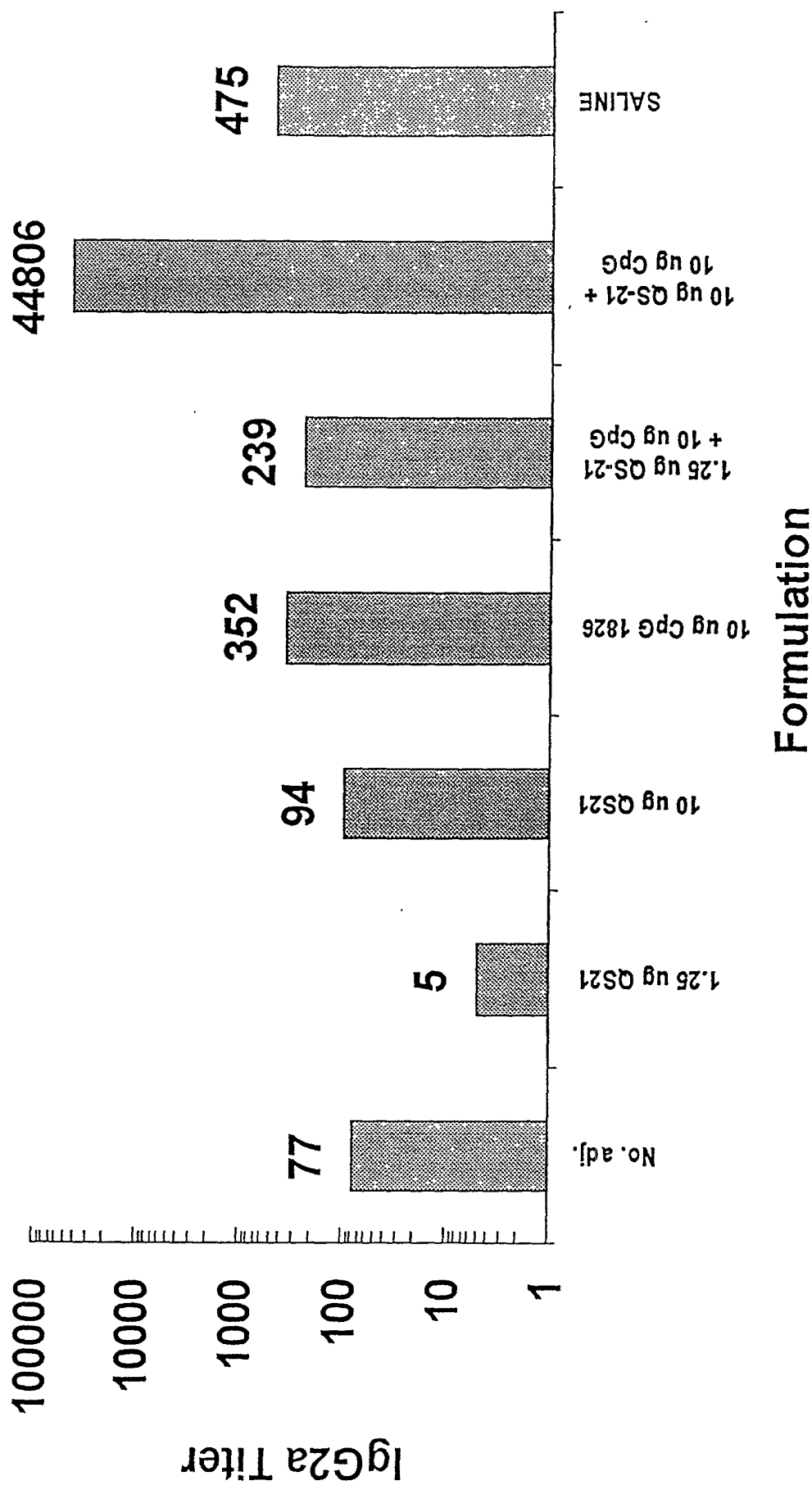
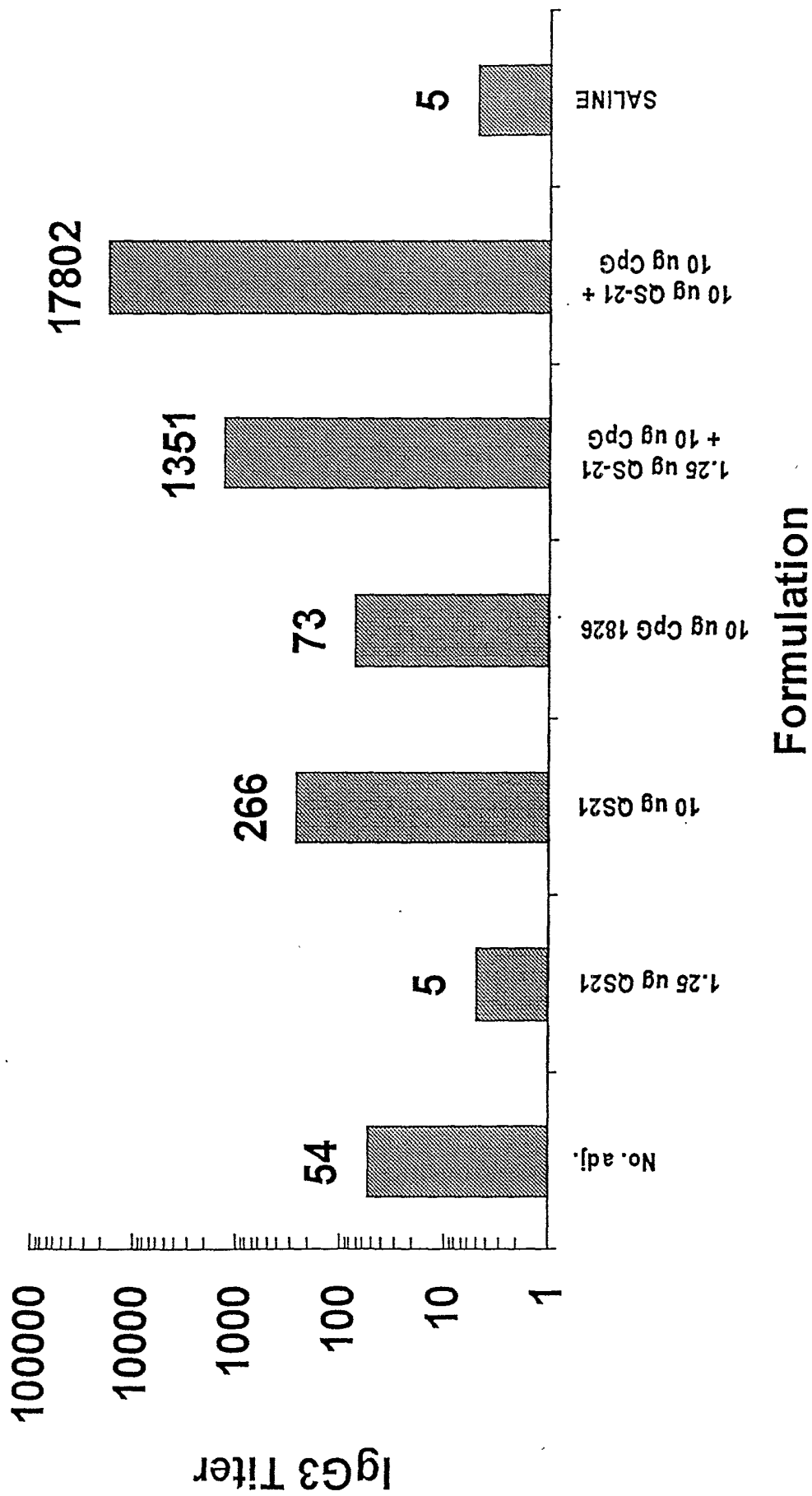


Figure 9



DECLARATION AND POWER OF ATTORNEY
(Attorney Docket No. 106.941.181)

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship is as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

COMPOSITIONS OF CPG AND SAPONIN ADJUVANTS AND USES THEREOF

the specification of which (check only one):

☒ [X] is attached hereto.

☐ [] was filed as United States Patent Application
Serial No. _____
on _____
and was amended
on _____
(if applicable)

☐ [] was filed as PCT Patent Application
Serial No. _____
on _____
and was amended under PCT Article 19
on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of the claims of this application in accordance with Title 37, Code of Federal Regulations, Sections 1.56(a) and 1.56(b).

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

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DATE OF DEPOSIT AUG-UST 6, 1999

**PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS
UNDER 35 U.S.C. §119(a)-(d) or 365(b):**

COUNTRY (if PCT indicate PCT)	APPLICATION NUMBER	DATE OF FILING	PRIORITY CLAIMED UNDER 35 U.S.C. §119(a)-(b) or 365(b) (YES/NO)
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I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional patent application(s) listed below:

APPLICATION NUMBER	DATE OF FILING	STATUS: (PENDING OR ABANDONED)
60/128608	April 8, 1999	
60/095913	August 10, 1998	

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DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. § 120 or 365(c):**

APPLICATION NUMBER	DATE OF FILING (day, month, year)	STATUS: (PATENTED, PENDING OR ABANDONED)
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POWER OF ATTORNEY: As named inventors, we hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith

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Wherefore I petition that letters patent be granted to me for the invention or discovery described and claimed in the attached specification and claims, and hereby subscribe my name to said specification and claims and to the foregoing declaration, power of attorney, and this petition.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Inventor's signature _____ Date _____

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